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Characterization and site-directed mutation of a novel aldo–keto reductase from *Lodderomyces elongisporus* NRRL YB-4239 with high production rate of ethyl (*R*)-4-chloro-3-hydroxybutanoate

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Abstract A novel aldo–keto reductase (LEK) from *Lodderomyces elongisporus* NRRL YB-4239 (ATCC 11503) was discovered by genome database mining for carbonyl reduction. LEK was overexpressed in *Escherichia coli* BL21 (DE3), purified to homogeneity and the catalytic properties were studied. Among the substrates, ethyl 4-chloro-3-oxobutanoate was converted to ethyl (*R*)-4-chloro-3- hydroxybutanoate (*(R)*-CHBE), an important pharmaceutical intermediate, with an excellent enantiomeric excess (e.e.) (>99 %). The mutants W28A and S209G obtained by site-directed mutation were identified with much higher molar conversion yields and lower Km values. Further, the constructed coenzyme regeneration system with glucose as co-substrate resulted in a yield of 100 %, an enantioselectivity of >99 %, and the calculated production rate of 56.51 mmol/L/H. These results indicated the potential of LEK for the industrial production of *(R)*-CHBE and other valuable chiral alcohols.

Keywords Aldo–keto reductase · Asymmetric reduction · Ethyl (*R*)-4-chloro-3- hydroxybutanoate

Introduction

Nowadays, enzyme-catalyzed biosynthesis becomes more and more attractive for the high enantioselectivity, mild and environmentally friendly reaction conditions. Enzymes for asymmetric reduction mainly include aldo–keto reductase (AKR), short-chain alcohol dehydrogenase/reductase (SDR), and medium-chain dehydrogenase families. Many AKRs and SDRs were developed for the production of enantiomerically pure secondary alcohols, which are important and valuable chiral synthons for chemical and pharmaceutical synthesis [1].

Optically active ethyl (*R*)-4-chloro-3- hydroxybutanoate ((*R*)-CHBE), for instance, is a key chiral intermediate in the enantioselective synthesis of L-ptomaine [2], macrolide A [3], atorvastatin calcium [4], (*R*)-γ-amino-8-hydroxy acetic acid (GABOB) [5], Negamycin [6], and 2,5-hexofibrin [7]. Asymmetric reduction of prochiral ketone ethyl 4-chloro-3-oxobutanoate (COBE) with AKR/SDR was demonstrated as an effective approach to produce (*R*)/(*S*)-CHBE with high yield and remarkable enantioselectivity [7]. Compared with (*S*)-specific enzymes, few (*R*)-specific carbonyl reductases were reported to produce (*R*)-CHBE because of laborious traditional screening from soil samples. Furthermore, most of (*R*)-specific carbonyl reductases reported had moderate yield or enantioselectivity <70 % [8]. Only two of them had both yield and enantioselectivity >90 % [9, 10], however, the catalytic process of which was time consuming and cannot be applied to industrial scale production.

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Recently, genome database mining has been proved as a more effective and promising approach to develop novel enzymes. AKRs comprise a large family of NAD(P)H-dependent oxidoreductases, with over 13,000 sequences available in Genbank and more than 200 crystal structures determined in Protein Data Bank. In the current study, a novel aldo–keto reductase from *Lodderomyces elongisporus* NRRL YB-4239 was discovered by genome database mining, based on the results of sequence alignment, conserved catalytic motif analysis and 3D molecular model simulation. After determined for the catalytic properties, key amino acids were chosen for further site-directed mutation. Two mutants of LEK were identified with much higher molar conversion yields and lower Km values. Furthermore, the highly efficient synthesis of (*R*)-CHBE was achieved with constructed cofactor regeneration system to evaluate the potential of LEK mutant for industrial application.

Materials and methods

Strains and materials

Lodderomyces elongisporus NRRL YB-4239 (ATCC 11503) was stored in the laboratory. The vectors pET28a (+), pET22b and pETDuet-1 were obtained from Novagen (Madison, WI, USA). The *Escherichia coli (E. coli)* strains DH5α and BL21 (DE3) were purchased from Stratagene (La Jolla, CA, USA). The restriction enzymes and polymerase used were purchased from Takara (Japan). All other chemicals were of reagent grade.

Gene cloning

Sense and antisense primers were designed according to the amino acids sequence of the putative protein LELG_05392 (GenBank Accession No. *XM_001523496.1*) from *Lodderomyces elongisporus*. The restriction sites of *Bam*HI and *Hin*dIII were introduced at the 5′ and 3′ ends, respectively. The nucleotide fragment was obtained by PCR using the genome of *Lodderomyces elongisporus* NRRL YB-4239 (ATCC 11503) as the template. The fragment was then digested and ligated with pET28a (+). After confirmation by sequencing, the vector pETLEK was transformed into *E. coli* BL21 (DE3).

Expression and purification

The expression of the recombinant protein was performed as described elsewhere [11]. To obtain the purified recombinant protein, the standard procedure was performed using an AKTA Prime system (Amersham-Pharmacia Biotech, Sweden) as previously described [11].

Enzyme activity assay and substrate spectrum determination

The enzyme activity of LEK was determined spectrophotometrically as follows. The assay mixture consisted of 100 mM phosphate buffer saline (pH 7.0), 0.1 mM NADPH, 5 mM substrate and 2 % (v/v) DMSO. The reactions were incubated at 30 °C and monitored for the decrease in absorbance at 340 nm. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol NADPH per minute. The substrates used for determination included a collection of aldehydes and ketones.

Effects of pH and temperature on enzyme activity and stability

A study on the effects of pH and temperature was performed with COBE as described [12].

Effects of metal ions on enzyme activity

To determine the effects of metal ions on enzyme activity, the recombinant enzyme LEK was incubated at 4 °C for 1 h with 1 mmol/L Ca^{2+}, Co^{2+}, Mn^{2+}, Li^{+}, Mg^{2+}, Na^{+}, Fe^{3+}, and Cu^{2+}, respectively, after which the residual activity was assayed.

Kinetic parameters

The apparent kinetic parameters for the reduction of COBE were calculated by measuring the enzyme activity at different COBE concentrations and fitting the data to the Michaelis–Menten equation.

Molecular modeling and site-directed mutagenesis

The molecular modeling of LEK was performed with the online program SWISS-MODEL (http://swissmodel.expasy.org) via the ExPASy web server, and the result was visualized using the program DeepView (Swiss Pdb-Viewer).

Site-directed mutagenesis was performed by overlap extension PCR with the following mutagenic primers: for W28A, 5′-TTGGGAACAATGCAAGCCACC-3′; for W28 M, 5′-GTTTGGGAACAGCGCAAGCCACCGAAG-3′; for L207F, 5′-TACTCTCCATTTGGCTCAACC-3′; for S209H, 5′-TCCATTGGGCCATACCGACTC-3′; and for S209G, 5′-ACTCTCCATTGGGCGGTACCGACTCGCCACTT-3′ (mutated codons are underlined). The first round of PCR was performed using LA Taq DNA polymerase with the mutagenic primers and sense/antisense primer of LEK. The second round of PCR was performed using Pyrobest DNA polymerase with the two PCR products as the template.

The PCR program consisted of the following steps: 95 °C for 3 min, 25 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min (1 min 20 s for the second round of PCR), followed by 72 °C for 10 min. The amplified fragment was digested with *Bam*HI and *Hin*dIII and then subcloned into the corresponding sites of pET28a (+). All mutations were confirmed by sequencing.

Construction of the plasmid for coexpression

The gene of glucose dehydrogenase (GDH) was obtained as previously described [13]. The restriction sites of *Bgl* II and *Xho* I were introduced at the 5′ and 3′ ends, respectively. The PCR product of the GDH gene was confirmed by sequencing and then ligated with pET22b. The expression and activity assay of GDH was performed as described in Sects. 2.3 and 2.4. The fragments of the LEK mutant gene and GDH were then digested with *Bam*HI/*Hin*dIII and *Bgl* II/*Xho* I, respectively, and then subcloned into the corresponding sites of pETDuet-1(106 for *Bam*HI, 998 for *Hin*dIII, 1160 for *Bgl* II, and 1973 for *Xho* I) within two independent reading frames. The constructed recombinant plasmid pETDuet-LEKM-GDH was confirmed by sequencing and then transformed into *E. coli* BL21.

Bioconversion of COBE to (*R*)-CHBE

Reaction mixtures containing 17.20 g/L *E. coli* cells harboring pETDuet-LEKS209G-GDH were incubated at 35 °C with stirring; the mixtures contained different concentrations of COBE ranging from 6.1 mM to 111 mM, 2 % (v/v) DMSO, and 97.5 mM glucose in 100 mM sodium acetate buffer (pH 6.0). The reaction with 17.20 g/L *E. coli* cells harboring pETLEKS209G and 12.75 g/L *E. coli* cells harboring pETGDH was performed under the same conditions. The molar conversion yield was determined at certain intervals during the catalysis.

The concentrations of COBE and CHBE after reaction as well as the optical purity of CHBE were analyzed as described previously [14].

Results and discussion

Genetic analysis and molecular modeling

The genome of *Lodderomyces elongisporus* NRRL YB-4239 (ATCC 11503) was sequenced recently, which meant there were lots of putative aldo–keto reductase genes to be screened and characterized. Desired catalytic properties could be discovered for the aldo–keto reductases from new resources such as the pathogen since no similar resources were reported. Instead of alignment with the sequences of reported enantioselective enzymes, genetic analysis and molecular modeling were used as the method of *Lodderomyces elongisporus* NRRL YB-4239 (ATCC 11503) genome database screening, which had been demonstrated effective in developing novel enzymes [12].

In the amino acids sequence of LEK, 25 highly conserved residues were found as the active sites in the aldo–keto reductases superfamily (see Online Resource 1). A web-based BLAST search (http://blast.ncbi.nlm.nih.gov/) revealed that LEK was significantly similar (over 40 % similarity) to (putative) aldo–keto reductases (see Online Resource 1), which suggested that LEK belongs to the aldo–keto reductases superfamily. The relations between LEK and homologous proteins were visualized by constructing a phylogeny tree (see Online Resource 2).

The molecular model of LEK (see Online Resource 3) exhibited a typical $(\alpha/\beta)_8$ barrel fold with 8 parallel β-strands and 8 α-helixes anti-parallel to the strand, which is highly conserved in the aldo–keto reductases superfamily [15]. Twenty-five conserved residues (26G, 27T, 28 W, 56D, 61Y, 82 K, 114H, 115 W, 154S, 155 N, 178Q, 204Y, 205S, 206P, 207L, 208G, 209S, 232A, 247L, 248P, 249 K, 250S, 255R, 258S, and 259 N) were located in the short loops between α-helixes and β-folds, among which the 4 residues (56D, 61Y, 82 K, and 114H) were composed of the highly conserved catalytic tetrad and 5 residues (56D, 155 N, 178Q, 255S, and 358R) composed of the conserved cofactor binding site. Therefore, the gene of LEK was chosen for the further study.

Cloning, expression and purification

To overexpress the gene of LEK, the vector pET28a (+) was selected to ensure the correct orientation and desired restriction sites. A His-Tag and an additional 28 amino acids were added to the N-terminal of LEK. After induction, the maximum protein expression was recorded to be over 40 %

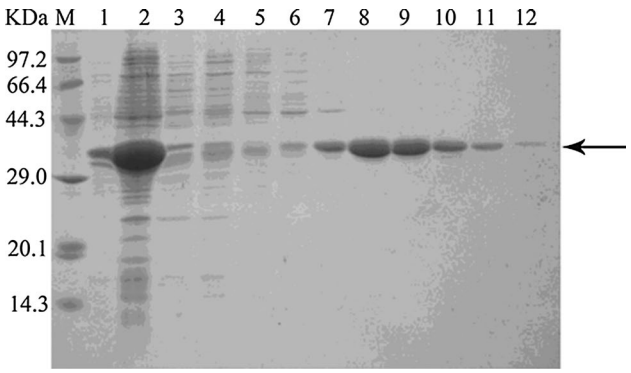


Fig. 1 Expression and purification of His-tagged LEK. *M* protein molecular weight maker, *Lane 1* the lysate before induction, *Lane 2* soluble fraction of lysate after induction, *Lane 3* the protein not combined to Ni–NTA, *Lane 4–12* eluates collected with 10, 20, 60, 100, 200, 250, 300, 400, and 500 mM imidazole

of the total protein expression. The apparent size of the protein was in good agreement with the calculated molecular weight of 36.3 kDa (Fig. 1). Elution with a linear gradient of imidazole (10–500 mM) was performed to obtain the His-tagged protein. The protein was mainly released by the elution buffer of 200–250 mM imidazole, and the apparent purity was over 90 % (Fig. 1). The yield of the purified protein was 5.3 mg, with 45 mg crude extracts as the starting material from the 100 ml culture medium. Although yeast cells were reported as the host to obtain aldo–keto reductases, *E. coli cell* was preferred because of less cost,higher biomass concentration,and shorter period of fermentation.

Substrate spectrum determination

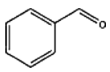
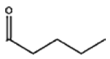
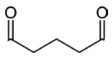
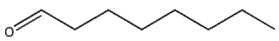
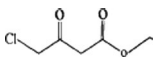
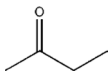
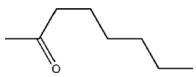
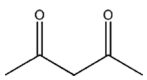
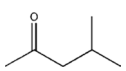
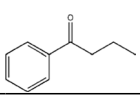
Aldehydes and ketones were chosen to determine the substrate specificity of LEK (Table 1), based on reports about substrate spectrum of AKR enzymes. NADPH was utilized as a cofactor, and no activity was observed with NADH.

High activities were detected for aldehydes while no reduction activity was observed for most of ketones. The activities for aldehydes decreased with the increasing carbon number, which was probably because of the space needed to access to the substrate binding site. The highest activity was detected for the diketone 2, 3-pentanedione, whereas no activity was observed for 2, 4-pentanedione, which suggested that LEK only catalyzed the β ketone group.

Effects of pH, temperature and metal ions on enzyme

Studies on the effects of pH, temperature and metal ions were performed using COBE as the substrate. The highest activity was obtained at pH 6.0, and more than 70 % of the

Table 1 Substrate spectrum in the reduction catalyzed by LEK

Reduction substrate	Structure	Enzyme activity (U/mg)	Relative activity (%)
Benzaldehyde		0.173 ± 0.001	87.7
n-Butyl aldehyde		0.190 ± 0.002	96.7
n-Valeral		0.146 ± 0.001	74.2
Glutaraldehyde		0.126 ± 0.001	64.1
Octanal		0.172 ± 0.004	87.4
2, 3-Butanedione		0.173 ± 0.003	87.8
2, 3-Pentanedione		0.196 ± 0.001	100
COBE		0.178 ± 0.004	90.7
2-Butanone		0 ± 0.001	0
2-Octanone		0 ± 0.001	0
3-Pentanone		0 ± 0.001	0
2, 4-Pentanedione		0 ± 0.001	0
4-methyl-2-Pentanone		0 ± 0.001	0
1-Phenyl-butanone		0 ± 0.001	0

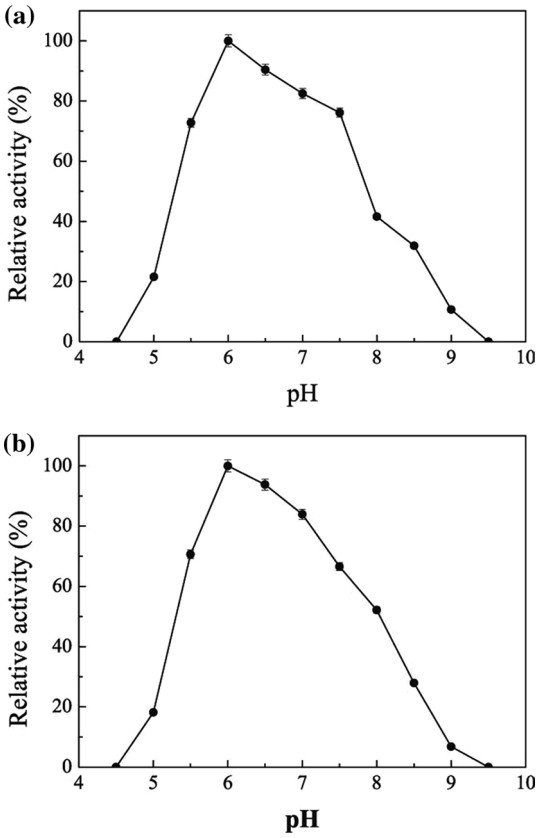


Fig. 2 Optimum pH and pH stability of LEK. **a** The optimum pH was determined by analysis of the activity at pHs from 4.5 to 9.5. **b** The effect of pH on enzyme stability was determined by comparing the relative activity of the enzyme (at 0.5 mg/ml) incubated at pHs ranging 4.5–9.5 at 4 °C for 60 min

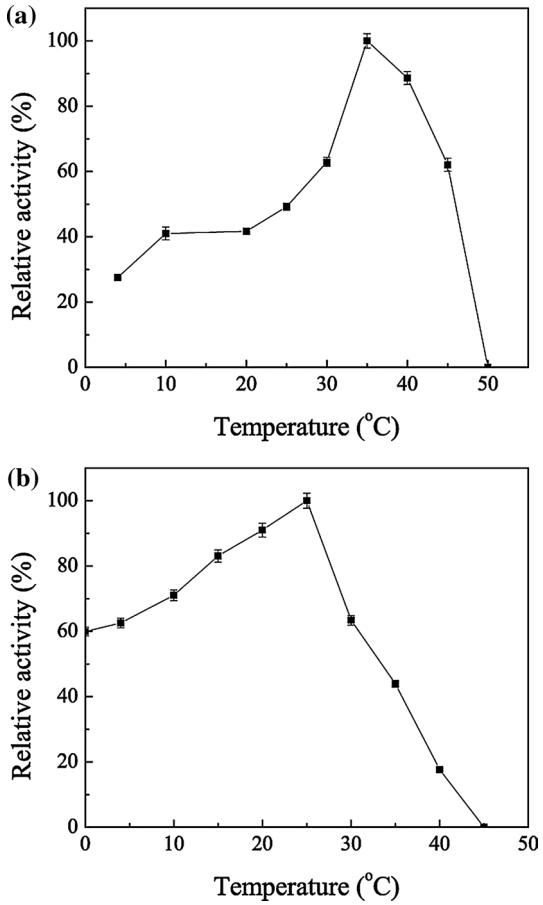


Fig. 3 Optimum temperature and thermal stability of LEK. **a** Activities were measured from 20 to 50 °C with the standard assay method to determine the optimum temperature. **b** The enzyme was incubated at temperatures ranging from 20 °C to 50 °C for 30 min, after which the residual activity was assayed

enzymatic activity was retained between pH 5.5 and 7.5 (Fig. 2a). After incubation at different pH levels ranging from 5.5 to 7.5 for 60 min, the enzyme activity remained over 65 % (Fig. 2b), suggesting that LEK would be stable in industrial processes under different pH conditions.

The highest enzyme activity was observed at 35 °C, and above 45 °C, the activity sharply decreased (Fig. 3a). The thermal stability of LEK was determined at different temperatures ranging from 10 to 40 °C. After incubation for 30 min, the enzyme activity remained 60 % at 30 °C and decreased to 20 % at 40 °C (Fig. 3b).

The effects of various metal ions on LEK activity was shown in Table 2. Mn^{2+}, Li^{+} and Ca^{2+} caused distinct increase in activity and it reached the maximum of 81.8 % with Mn^{2+}. The enzyme activity was inhibited significantly when Co^{2+}, Mg^{2+}, or Na^{+} was added to the reaction mixture, respectively. Fe^{3+} and Cu^{2+} deactivated the enzyme shortly after incubation with no activity remained. The results reveal that Mn^{2+}, Li^{+} and Ca^{2+} could be used for higher LEK activity, and Co^{2+}, Mg^{2+}, Na^{+}, Fe^{3+}, or Cu^{2+} should be avoided in the reaction mixture.

Table 2 The effects of various metal ions

Metal ion	Relative activity (%)
None	100.0
Mn^{2+}	181.8 ± 0.1
Li^{+}	125.4 ± 0.2
Ca^{2+}	121.8 ± 0.1
Co^{2+}	54.5 ± 0.3
Na^{+}	50.9 ± 0.1
Mg^{2+}	45.4 ± 0.4
Cu^{2+}	0
Fe^{3+}	0

Apparent kinetic and catalytic properties of LEK and mutants

The characteristics of the reported enzymes producing (*R*)-CHBE are summarized in Table 3. ARI, CmAR, ALR and

Table 3 Comparison of the characteristics of enzymes producing (*R*)-CHBE [5, 9, 10, 16, 17]

Enzyme	ARI	CmAR	BYueD	ALR	Gox2036	LEK
Source	*Sporobolomyces salmonicolor*	*Candida magnoliae*	*Bacillus sp.*	*Sporobolomyces salmonicolor*	*Gluconobacter oxydans*	*Lodderomyces elongisporus*
e.e. for (*R*)-isomer	100	100	100	99	100	99
Molecular weight (kDa)	37	35	30.7	Not reported	28	36.3
Protein family	AKR	AKR	CAR	ALR	SDR	AKR
Cofactor	NADPH	NADPH	NADPH	NADPH	NADH	NADPH
Optimum pH	7	7	7.5–8.0	6.5	6.0	6.0
Optimum temperature (°C)	60	40	45–50	37	30	35
Km for COBE (mM)	0.36	2.9	0.70	Not reported	0.77	37.01

LEK were from distinct yeast species while BYueD and Gox2036 were of bacterial origin. ARI, CmAR and LEK belong to the aldo–keto reductase (AKR) family. BYueD belongs to the carbonyl reductase (CAR) family, and ALR belongs to aldehyde reductase (ALR) family. Gox2036 belongs to the short-chain dehydrogenase/reductase (SDR) family. All of the enzymes produced (*R*)-CHBE with enantioselectivity >99 % e.e., and most of them preferred NADPH as cofactor. The Km value of LEK for COBE was 37.01 mM, the highest compared with other enzymes, which means the lowest affinity with the substrate. The Kcat value of LEK for COBE was 9.96 μmol min^{-1} and Kcat/Km was 0.27 μmol mM^{-1} min^{-1}. Therefore, site-directed mutation was performed for better properties.

Highly conserved amino acids usually play an important role in the catalysis process and in maintaining the active structure of catalysts, the mutagenesis of which should be avoided. Therefore, based on molecular modeling and multiple sequence alignment, three residues (28 W, 207L and 209S) with less conservatism were chosen for site-directed mutation, which were supposed to give rise to the molar conversion yield and substrate specificity in the short loops at the C-terminal of the barrel structure.

The catalytic properties of mutants were determined and compared with LEK. Activities for the mutants W28A, W28 M, S209H and S209G changed slightly, but decreased sharply to 15.4 % for L207F, which means that 207L (Online Resource 3) is a crucial amino acid to the reduction activity and probably the side chain of phenylalanine (F) blocked the binding with COBE.

The molar conversion yields of COBE were 99.0 and 87.9 % for S209G and W28A, respectively (Table 4), higher than 78.0 % for LEK. The yields for other mutants were slightly lower than that of LEK. The e.e. value of W28 M decreased to approximately 85 %, while the values of other mutants were all above 99 % for the (*R*)-isomer. The apparent Km values of S209G and W28A were determined to be 6.05 and 14.08 mM, respectively, which means that the mutants have remarkably higher affinity to the substrate than LEK. The lower Km value is also advantageous in the industrial process because high concentration of substrates can destabilize the enzymes.

The results suggested that 209S is important in substrate binding and catalysis. When serine was replaced

Table 4 Comparison of the properties of LEK and two mutants

Enzyme	LEK	W28A	S209G
Relative activity (%)	100.0	95.7	101.0
Molar conversion yield (%)	78.0	87.9	99.0
e.e. (%)	>99	>99	>99
Km for COBE (mM)	37.01	14.08	6.05
V_{max} (μmol/min/mg)	0.67	0.074	0.55

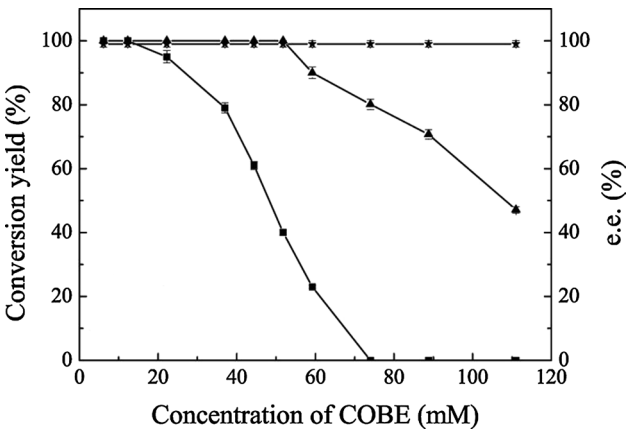


Fig. 4 The effects of substrate concentration on reaction catalyzed by constructed cofactor regeneration system. *Star* indicates e.e. values; *Triangle* indicates cells harboring pETLEKS209G and pETGDH, respectively; *Square* indicates cells harboring pETDuet-LEKS209G-GDH

Table 5 The production rate of (*R*)/(*S*)-CHBE [5–7, 10, 18–20]

Source/ Biocatalyst	*Candida magnolia*	Baker's yeast	*Lactobacillus kefir*	*Sporobolomyces salmonicolor*	*Gluconobacter oxydans*	*Lactobacillus fermentum*	LEKS209G/ GDH
Isomer	*S*	*S*	*S*	*R*	*R*	*R*	*R*
e.e.	96	97	99.5	86	100	98	99
Production rate (mmol/L/H)	9	3.4	85.7	8.2	5.8	0.5	56.51

by glycine at 209 (Online Resource 3), the hydrophobicity was enhanced, and the pocket binding to the substrate was broader without the side chain of serine, which might be the reason for the increased conversion yield and higher affinity to COBE. However, when serine was replaced with histidine, the side chain became a heterocyclic group, acting more as a blockade, and the charge became positive, which most likely resulted in the decreased conversion yield of S209H.

The results also revealed that 28 W is a crucial amino acid involved in conversion yield, enantioselectivity and affinity to the substrate. When tryptophan was replaced by hydrophobic alanine with smaller size at 28 (Online Resource 3), the substrate was easier to access to the binding site, which resulted in the improved conversion yield. When tryptophan was replaced by methionine at 28, the enantioselectivity observably decreased probably because the side chain of M affected the configuration of the product during the catalysis process.

Asymmetric bioconversion of COBE to (*R*)-CHBE with cofactor regeneration system

For cells harboring pETDuet-LEKS209G-GDH as the catalyst, the molar conversion yield reached 100 % with 12.3 mM COBE, 95 % with 22.2 mM COBE, and decreased sharply with substrates containing more than 37 mM COBE (below 80 %, Fig. 4). The conversion yield reached 50 % in 1 h and the maximum yield in 3.5 h with 22.2 mM COBE. The calculated production efficiency was 6.03 mmol/L/H, which is similar to previously reported results (Table 5).

For cells harboring pETLEKS209G and pETGDH, respectively, as the cofactor regeneration system, the conversion yield reached 100 % with 51.8 mM COBE, 90 % with 59.2 mM COBE, and decreased sharply with substrates containing more than 74 mM COBE (below 80 %, Fig. 4). The conversion yield reached its maximum in 55 min with 51.8 mM COBE. The calculated production rate was 56.51 mmol/L/H, which is the highest in the reported asymmetric bioconversion of (*R*)-CHBE (Table 5). The e.e. values were also determined because instability and degradation of COBE in an aqueous monophase system could result in differences in conversion yield. In all cases, the e.e. values of the product for the (*R*)-isomer were over 99 %.

The expression level of pETDuet-LEKS209G-GDH was only approximately 10 % of the total protein for each recombinant protein (data not shown), much lower than the levels expressed with individual plasmids (40 % for LEKS209G and 20 % for GDH), which might resulted in the lower production rate. In the mean time, it was also difficult to ensure that two enzymes in the same host cell exhibit equal levels of activity, which restricts the application of the coexpression method.

Conclusions

In conclusion, the gene of LEK from *Lodderomyces elongisporus* NRRL YB-4239 (ATCC 11503) was cloned and characterized as an asymmetric aldo–keto reductase. The substrate spectrum and catalytic characteristics were investigated. Site-directed mutagenesis was performed to obtain improved properties. Two mutants were identified with higher molar conversion yields and lower Km values. The *E. coli* cells harboring of pETLEKS209G and pETGDH were shown to provide an efficient cofactor regeneration system for the synthesis of optically pure (*R*)-CHBE with a satisfactory e.e. value, excellent of molar conversion yield and high production rate, which could be further used for the industrial production of (*R*)-CHBE.

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Ethical statement The experiments comply with the current laws of China in which they were performed.

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